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XENOGENOUS FERTILIZATION OF SQUIRREL MONKEY AND GOLDEN HAMSTER OOCYTES

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XENOGENOUS FERTILIZATION OF SQUIRREL MONKEY AND GOLDEN HAMSTER OOCYTES

By

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ABSTRACT

XENOGENOUS FERTILIZATION OF SQUIRREL MONKEY
AND GOLDEN HAMSTER OOCYTES

by

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The ability of the rabbit oviduct to support the fertilization of squirrel monkey and hamster ova, namely, xenogenous fertilization, was studied. The following parameters were examined: sperm concentration, day of rabbit pseudopregnancy, time of recovery and number of oocytes deposited into the rabbit oviduct.

Hamster ova recovered from superovulated females were placed in the oviduct of day 1, 2, 4, 7 or 10 pseudopregnant rabbits and the oviducts were inseminated. Epididymal sperm in concentrations of 10^6, 10^7, 10^8 or 10^9 sperm/ml were tested. The ova were then recovered from the rabbit oviduct 28, 29, 30 or 32 hours later.

Squirrel monkey ova recovered by laparoscopic follicular aspiration from gonadotropin stimulated ovaries were placed in the oviduct of 1, 2 or 3 day pseudopregnant rabbits. These oviducts were inseminated with 10^6, 10^7 or 10^8 sperm/ml and recovered 12 to 98 hours after insemination. Fertilization of squirrel monkey and hamster ova were judged by the presence of 2 polar bodies and 2 pronuclei or cleavage.

Xenogenous fertilization rates for squirrel monkey and hamster ova were 25/79 (31.6%) and 119/198 (60.1%) respectively with 3/25

(12.0%) and 38/119 (31.9%) of the fertilized squirrel monkey and hamster ova cleaving, respectively. Day of rabbit pseudopregnancy and sperm concentration had no effect on the fertilization or cleavage rates of hamster and squirrel monkey ova. Cleavage time was observed to be at least 28 hours for hamster ova xenogenously fertilized and at least 31 hours for xenogenously fertilized squirrel monkey ova. These developmental times correspond to normal *in vivo* developmental rates. The number of hamster ova deposited into the rabbit oviduct had no effect on fertilization.

To my mother

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INTRODUCTION

The study of the fertilization process can benefit both the prevention of unwanted human pregnancies and the productivity of domestic animals raised for food consumption. The ability to efficiently generate normal embryos has application to: 1) some types of human infertility; 2) embryo transfer in domestic and endangered species; 3) screening of potentially teratologic, mutagenic and toxicologic compounds; 4) testing of fertility and infertility drugs and 5) scientific research.

Presently there are five possible methods for the generation of mammalian embryos other than natural fertilization: 1) in vitro fertilization; 2) monogametic fusion; 3) blastomere separation; 4) parthanogenic activation and 5) xenogenous fertilization. The latter is the fertilization of oocytes with homologous sperm in the oviduct of a heterologous species. The term xenogenous comes from the Greek roots "xeno" (foreign) and "genous" (nuture). Possibly the oviducts of the pseudopregnant rabbit would constitute a suitable environment for normal fertilization and development of hamster and squirrel monkey gametes. This possibility was investigated.

LITERATURE REVIEW

In Vitro Fertilization

The discovery of the need for sperm to be incubated in the female reproductive tract prior to fertilization, i.e. capacitation (Chang, 1951; Austin, 1951) was followed by many reports of *in vitro* fertilization (Table 1). Live births have resulted from embryo transfer of *in vitro* fertilized ova has been accomplished in mice, rats, rabbits (Brackett, 1979) and man (Steptoe and Edwards, 1978; Lopata *et al.*, 1980).

In vitro fertilization of golden hamster (*Mesocricetus auratus*) ova was first accomplished by incubating ova and oviductal components with sperm recovered from the uteri of mated females or the epidydimi of males. Sperm, collected from the uterus, fertilized a higher percentage of the hamster ova (Yanagimachi and Chang, 1963, 1964). This higher fertilization rate can be attributed to the female tract and not male accessory sex gland secretions, since male secretions decreased the fertilizability of epididymal sperm *in vitro* (Tsunoda and Chang, 1977). The penetration of ova by sperm was slower *in vitro* than *in vivo* (Yanagimachi, 1966) and development of hamster ova fertiilized *in vitro* was halted at the two cell stage (Yanagimachi and Chang, 1964). A scanning electron microscopic examination of the ultrastructure of hamster ova fertilized *in vitro* or *in vivo* showed

TABLE 1

In Vitro Fertilization of Mammalian Ova

Species	Investigators
Guinea Pig	Yanagimachi, 1970b, 1972
Rabbit	Chang, 1954; Dauzier and Thibault, 1959
Mouse	Whittingham, 1968; Pavlok, 1968
Rat	Toyoda and Chang, 1974
Gerbil	Noske, 1972
Golden Hamster	Yanagimachi and Chang, 1963, 1964
Chinese Hamster	Pickworth and Chang, 1969
Cat	Hamner *et al.*, 1970; Bowen, 1977
Dog	Mahi and Yanagimachi, 1976
Pig	Harns and Smith, 1970; Iritani *et al.*, 1975; Iritani *et al.*, 1978
Sheep	Dauzier and Thibault, 1959; Kraemer, 1966; Biondioli and Wright, 1980
Cow	Bregalla *et al.*, 1974; Iritani and Niwa, 1977; Bracket *et al.*, 1977, 1978, 1980
Squirrel Monkey	Cline *et al.*, 1972, Johnson *et al.*, 1972; Gould *et al.*, 1973; Kuehl and Dukelow, 1975, 1979
Baboon	Kraemer *et al.*, 1979
Man	Blandau, 1980

that the mode of sperm binding to the vitelline membrane was different. For example, *in vitro*, the anterior tip of the sperm appears to bind to the vitellus first while *in vivo* the post acrosomal collar binds to the vitellus first (Shalgi and Phillips, 1980). Despite these differences there have been no other reports citing differences in the early events of fertilization between *in vivo* and *in vitro* systems and the *in vitro* system for hamster fertilization has been extensively used to study sperm capacitation and other processes in fertilization.

In vitro fertilization of hamster ova can occur in an environment where the osmolality ranges from 232 to 452 mosmolal with maximum fertilization at 292 to 390 mosmolal (Miyamoto and Chang, 1973). *In vitro* fertilization of hamster ova can occur in media ranging in pH from 6.7 to 8.7 with optimum fertilization at a pH of 6.8 to 8.2 (Miyamoto *et al.*, 1944). Sperm concentration is important for *in vitro* fertilization. The optimum sperm concentration for *in vitro* fertilization of hamster ova was reported to be $2x10^7$ sperm/ml with declining fertilization rates at greater or lesser sperm concentrations (Talbot *et al.*, 1974).

Ovum concentration also affects the rate of *in vitro* fertilization in a defined medium (Niwa *et al.*, 1980). The importance of ovum concentration on *in vitro* fertilization is not due to the ova themselves but rather their follicular constituents (Barros, 1968). Fertilization rates of hamster ova that were washed to remove all the follicular fluid was decreased (Barros and Austin, 1966, 1967) and hamster ova devoid of the cumulus cells were not fertilized (Gwatkin

et al., 1972). The contribution of the follicular fluid, cumulus cells and cumulus matrix to fertilization can be mimicked with: mouse or rat oviductal fluid (Barros, 1968); heat inactivated bovine follicular fluid (Gwatkin and Anderson, 1969; Yanagimachi, 1969a), mouse or rat follicular fluid (Yanagimachi, 1969b); and heat inactivated hamster (Miyamoto and Chang, 1972), rat, guinea pig, rabbit, bull or human sera (Barros and Garavagno, 1970; Yanagimachi, 1970a). Therefore, for in vitro fertilization of hamster ova to occur, there must be present a nonspecific factor(s) to facilitate sperm capacitation. This factor may be an albumin-like compound, since hamster sperm have been capacitated in vitro in defined media to which bovine serum albumin was added. Albumin, however, did not cause the acrosome reaction in these spermatozoa and other factors may be involved (Bavister, 1969, 1973).

Sperm will attach to the zona pellucida of squirrel monkey ova in vitro (Johnson et al., 1972). Others have demonstrated that fertilization of squirrel monkey will occur in vitro and these fertilized ova will develop to the 2 cell (Cline et al., 1972; Gould et al., 1973), 4 cell (Kuehl and Dukelow, 1975) and 8 cell stages (Kuehl and Dukelow, 1979). The time of development of in vitro fertilized ova of squirrel monkeys have been shown to be similar to that occurring in vivo in the baboon, rhesus monkey and human embryos, i.e. second polar body extrusion 6-22 hours after insemination; first cleavage 20-40 hours after insemination; second cleavage 46-52 hours after insemination and third cleavage 52-74 hours after insemination (Kuehl and Dukelow, 1979).

Monogametic Fusion

The production of embryos by fusion of two homologous oocytes is termed monogametic fusion. This has been accomplished in mice by fusing two zona-free oocytes using inactivated sendai vivus to activate the fusion process (Soupart et al., 1977, 1979). Zona-free mouse ova were incubated for 3 minutes with ultraviolet light inactivated sendai vivus. The two ova were then placed together to allow fusion. One hour after fusion occurred it was observed that the cortical granules were extruded from the fused oocytes and meiosis resumed. Five hours after fusion, two second polar bodies were extruded (Soupart, 1977). When fused mouse ova were cultured for 5.5 days the zygotes developed to the blastocyst stage with 120 to 130 nuclei. The development of the zygote was dependent on the three dimensional integrity of the blastomeres. If this was lost, development ceased at the eight cell stage. When three dimensional integrity was maintained by placing the blastomeres in an empty rabbit zona pellucida, development proceeded (Soupart et al., 1979). Thus, the fusion of two oocytes restored the diploid number of chromosomes and resulted in the events similar to those which occur after sperm-ovum fusion during the fertilization process.

Blastomere Separation

Separation of the blastomeres of two cell rat embryos and transfer of these blastomeres to pregnant recipients resulted in the development of those embryos to the egg cylinder stage (Nicholas and Mall, 1942). In rabbits (Seidel, 1952) and mice (Tarkowski, 1959)

destruction of one of the blastomeres of a two cell embryo and subsequent transfer of the remaining blastomeres can result in the birth of live offspring. Thus, each blastomere of an early developing zygote possesses the potential for complete development. The separation of the blastomeres of an early developing zygote could be used to increase the number of embryos produced by natural fertilization.

Blastomere separation and transfer of the resultant monozygotic twins has been accomplished in mice (Moustafa and Hahn, 1978), sheep (Willadsen, 1979, 1980) and cattle (Willadsen et al., 1981). Early developing embryos are collected and the zona pellucida are enzymatically or mechanically removed. The blastomeres are then mechanically separated with fine needles and placed in empty zona pellucida. In mice the embryos were transferred to recipient females (Moustafa and Hahn, 1978) but in the domestic species the embryos were embedded in a small cylinder of 1% agar in 0.9% NaCl. This agar chip is then encapsulated in a larger 1.2% agar shell. The embryos are then allowed to develop to the late morula or early blastocyst stage in a ligated oviduct of a ewe. The embryos are recovered and transferred to synchronized recipients (Willadsen, 1981). The results of blastomere separation and transfer of these species is shown in Table 2.

When damage due to micromanipulation was minimized and the embryos were separated into sets of blastomeres of equal cell number, the success of transfer did not differ significantly from normal embryo transfer results (Willadsen, 1980; Willadsen et al., 1981). However, when eight cell cattle embryos were divided into halves (2

TABLE 2

The Viability of Embryos Produced by Blastomere Separation

Species (reference)	Stage of Separation	Number of Embryos Transferred	Number Born (%)	Sets of Monozygotic Twins Born
Mice (Moustafa and Hahn, 1978)	8-16 cell	40	30 (75)	8
Sheep (Willadsen, 1979)	2-8 cell[+]	32	20 (62.5)	5
Sheep (Willadsen, 1980)	2-8 cell[+]	30	25 (83.3)	2
Cattle (Willadsen et al., 1981)	8 cell	50	30 (60.0)	10

[+]No difference was found in the viability of embryos derived from 2, 4 or 8 cell embryos.

pairs of 4 cell embryos) or quarters (4 pairs of 2 cell embryos) the survivial of the embryo was significantly decreased; i.e., 21 births out of 28 transfer verses 9 births out of 22 transfers, respectively (Willadsen et al., 1981).

The blastomere separation method represents a means of increasing the number of identical embryos produced by natural fertilization which can be used as a research tool or for enhancing fertility.

Xenogenous Embryo Culture

The ability of the female genital tract to support development of embryos of other species may be used as a means of culturing embryos or to increase the fecundity of endangered species (Durrant and Benirschke, 1981). Transfer of embryos between species within the same genus has been successful. To illustrate, Bunch et al. (1977) achieved three pregnancies which resulted in two live births by transferring ten fertilized mouflon ova (Ovis musima) to domestic sheep (O. aries). Intergeneric transfers, however, have not met with such success.

Attempts at reciprocal transfer between sheep and goats have not been successful (Lopyrin et al., 1951). In most cases, fertilized sheep ova transferred to synchronized goat uteri, will continue to develop for only 45 days. Fertilized goat ova transferred to synchronized sheep uteri will only develop for 22 to 27 days (Warwick and Berry, 1949; Hancock and McGovern, 1968). Failure of goat-sheep reciprocal transfer may be attributed to differences in length of the gestation between these species. Goats, with the longer gestation,

may be receiving sheep embryos which are physiologically older (Hancock and McGovern, 1968).

Attempts at reciprocal transfer between rats and hamsters has also met with limited success. Two days post-estrus, fertilized hamster ova were transferred to rats pseudopregnant for three days. Twenty-six of the 68 hamster ova transferred (38.2%) implanted but degeneration was observed. No implantation was observed when rat ova were transferred to hamsters (Blaha and DeFeo, 1964).

Reciprocal transfer of embryos between rats and mice resulted in development of 2 to 8 cell ova to the morulae (Beyer and Zeilmaker, 1973) and blastocyst stages (Beyer and Zeilmaker, 1973; Tarkowski, 1962). When reciprocal transfers of rat and mouse blastocysts were attempted, the blastocysts continued to develop (Zeilmaker, 1971) and a decidual reaction occurred (Tarkowski, 1962; Copp and Rossant, 1978). No blastocysts were attached to the recipients endometrium (Tarkowski, 1962) and rat embryo development ceased at the egg cylinder stage (Tarkowski, 1962; Copp and Rossant, 1978). Mouse embryos transferred to irradiated rat uteri, however, continued to develop to the stage of a 7 day old embryo (Zeilmaker, 1971). Failure of rat embryos to develop in mouse uteri did not result from abnormal embryo development since retransfer of rat embryos from the mouse to rat resulted in pregnancy and live birth (Beyer and Zeilmaker, 1973).

Although interspecific embryo transfer has not been proven successful in yielding live offspring from the recipient mother, the fact that early embryonic development can occur in the female genital tract

of a heterologous species could mean the female genital tract could be a site for embryo culture. Briones and Beaty (1954) attempted reciprocal transfer of fertilized ova among rats, mice, guinea pig, and rabbits. Nine of the possible twelve reciprocal transfers were attempted. Continued development of transferred ova was observed in: mouse ova in rabbit oviducts, rat uteri and guinea pig uteri; rabbit ova in mouse uteri, rat ovarian capsule, rat uteri and guinea pig uteri.

The most extensively utilized environment for embryonic culture has been oviducts of rabbits. This environment is capable of supporting the early embryonic development of: mice (Brinster and Ten Broeck, 1969); rats (Yoshinaga and Adams, 1967); snowshoe hares (Change, 1965); ferrets (Chang, 1966; Chang et al., 1971); cows (Hafez and Sougle, 1963; Adams et al., 1968; Sreenan et al., 1968; Sreenan and Scanlon, 1968; Lawson et al., 1972); sheep (Averill et al., 1955; Adams et al., 1961; Hunter et al., 1962; Adams et al., 1968; Lawson et al., 1972) and horses (Allen et al., 1976). A summary of the laboratory animal and domestic animal ova cultured in rabbit oviducts can be found in Tables 3 and 4, respectively.

Mouse embryo development in rabbit oviducts depends on the stage of development of the embryo when transferred. One cell mouse embryos were unable to develop whereas two cell ova developed to the blastocyst stage (Brinster and Ten Broeck, 1969). Sheep embryo development in the rabbit oviduct was not dependent on the stage of embryo development prior to transfer (Lawson et al., 1972). The hormonal stage of

TABLE 3

The Culture of Laboratory Animal Ova in the Rabbit Oviduct

Ovum Donor	Stage of Development at Transfer	Time in Rabbit Oviduct	Stage of Post-transfer Development	Investigator
Mouse	1 Cell	5 Days	None recovered	Brinster and Ten Broeck, 1969
Mouse	2 Cell	4 Days	Blastocyst	Brinster and Ten Broeck, 1969
Rat	Blastocyst	48 hours	Zona Pellucida Free Blastocyst[1,2]	Yoshinaga and Adams, 1967
Ferret	2-4 Cell	2 Days	8-12 cell	Chang, 1966
Ferret	Blastocyst	2-3 Days	Expanded Blastocyst[1]	Chang, 1971
Snowshoe Hare	2 Cell	6 Days	Blastocyst	Chang, 1965

[1]Retransfer after recovery from the rabbit resulted in pregnancy.

[2]Retransfer after recovery from the rabbit resulted in a live birth.

TABLE 4

The Culture of Domestic Animal Ova in the Rabbit Oviduct

Ovum Donor	Stage of Development at Transfer	Time in Rabbit Oviduct	Stage of Post-transfer Development	Investigator
Bovine	5-16 Cell	4 days	Blastocyst	Sreenan and Scanlon, 1968
	16-32 Cell	2-3 days	Blastocyst	Hafez and Sugie, 1963
	1-8 Cell	2-4 days	Morula to Blastocyst[1]	Lawson et al., 1972
	2-10 Cell	46-95 hrs	10-64 Cell	Sreenan et al., 1968
	8-32 Cell	3-4 days	Blastocyst	Adams et al., 1968
Ovine	2-12 Cell	4-5 days	Morula to Blastocyst[1]	Averill et al., 1955
	1-8 Cell	4-5 days	Morula to Blastocyst[1]	Adams et al., 1961
	2-8 Cell	102-108 hrs	Morula to Blastocyst	Hunter et al., 1962
	8-32 Cell	4-7 days	Blastocyst	Adams et al., 1968
	2-16 Cell	3-7 days	Morula to Blastocyst[1]	Lawson et al., 1972
Equine	32 Cell to expanding blastocyst	40-49 hrs	Early blastocyst to hatched blastocyst[1]	Allen et al., 1976

[1]Retransfer of embryos after recovery from rabbit resulted in pregnancy and a live birth.

the rabbit, did not effect development of sheep (Lawson et al., 1972) or mouse (Brinster and Ten Broeck, 1969) embryos.

Culture of embryos in a heterologous species is a means of maintaining embryos in later stages of development and also represents a means of embryo incubation during transport to the location of recipients. Sheep ova have been transported from England to South Africa (Adams et al., 1961; Hunter et al., 1962) and horse embryos have been transported from England to Poland (Allen et al., 1976) where they were transferred to synchronized recipients and live births subsequently resulted.

Xenogenous Fertilization

Xenogenous fertilization was first accomplished with bovine ova and sperm in the rabbit oviduct (Umbaugh, 1949). Subsequently, bovine ova have been xenogenously fertilized in: estrous ewes (Sreenan, 1970); prepubertal guilts (Shea et al., 1976; Bedirian et al., 1975); estrous rabbits (Trounson et al., 1977) and pseudopregnant rabbits (Hirst et al., 1981). In addition, porcine gametes have been fertilized in pseudopregnant rabbits (Hirst et al., 1981). Attempts to fertilize human ova in oviducts of the estrous rabbits and rhesus monkeys have failed (Edwards et al., 1966). A summary of published research on xenogenous fertilization is shown in Table 5.

Trounson et al. (1977) was able to fertilize bovine ova in the estrus rabbit oviduct, where as Sreenan (1970) could not. Trounson et al. (1977), however, observed cleavage of bovine ova when placed in the estrus rabbit oviduct with no bull sperm. Trounson et al. (1977)

TABLE 5

Xenogenous Fertilization of Mammalian Ova

Species	Recipient Oviduct	Reference	Ova Transferred	Ova Recovered (%)	Ova Fertilized (%)
Bovine	rabbit	Umbaugh, 1949	59	24 (40.7)	3 (12.5)
Bovine	pseudopregnant rabbit	Hirst et al., 1981	582	261 (44.8)	35 (13.4)
Bovine	estrous rabbit	Trouson et al., 1977	491	337 (68.6)	40 (11.9)
Bovine	estrous rabbit	Sreenan, 1970	82	—	—
Bovine	estrous ewe	Sreenan, 1970	375	198 (52.8)	17 (8.6)
Bovine	cycling ewe	Shea et al., 1976	24	16 (66.7)	0 (0)
Bovine	prepubertal gilt	Shea et al., 1976	67	29 (43.3)	5 (17.2)
Bovine	prepubertal gilt	Bederian et al., 1975	70	27 (38.6)	6 (22.2)
Porcine	pseudopregnant rabbit	Hirst et al., 1981	410	148 (36.1)	3 (2.0)
Human	estrous rabbit	Edwards et al., 1969	20	12 (60.0)	0 (0)
Human	rhesus monkey	Edwards et al., 1969	67	2 (3.0)	0 (0)

did not examine his embryos for sperm penetration. Thus, the fertilization reported might be due to parthenogenic development, as suggested by the investigator. Hirst et al. (1981) was able to show fertilization of bovine ova in the oviduct of a pseudopregnant rabbit. Parthenogenic development was less likely becuase of the observation of sperm penetration (Hirst, personal communication).

The importance of the hormonal state of the animal providing the incubating oviduct is emphasized by the ability of an estrous ewe to support fertilization of bovine gametes (Sreenan, 1970) while ewes at other stages of the cycle did not support fertilization of bovine gametes (Shea et al., 1974). Parthenogenesis can be ruled out in the study because non-inseminated controls did not show early embryonic development (Sreenan, 1970).

MATERIALS AND METHODS

Animal Care

Squirrel monkeys of the Bolivian type and Guyanan type were obtained from Primate Imports Corp. (Port Washington, New York) and South American Primates (Miami, Florida), respectively. They were housed in groups of six, in stainless steel, flush-type, cages from October through June. During the summer, June through September, they were housed outdoors, in groups of 50, in 4 outdoor colonies (Jarosz and Dukelow, 1976). The animals were fed with High Protein Monkey Chow, Jumbo 5047 (Ralston Purina Co., St. Louis, Missouri) and water ad libitum and fruit as a diet supplement. While being housed indoors, they were exposed to 12h:12h light:dark (0600 hrs to 1800 hrs light) cycle using fluorescent lighting.

The golden hamsters were obtained from the State of Michigan. The hamsters were housed in groups of six in plastic cages with stainless steel tops with ground corn cobs as bedding (San-I-Cell, Paxton Processing Co., Paxton, Illinois). Waynes Laboratory Animal Diet (Allied Mills Inc., Chicago, Illinois) and water was fed ad libitum. The animals were exposed to a light/dark cycle of 14:10 hrs (0600 hr to 1800 hr lights on).

Rabbits were obtained from local breeders and housed singly in stainless steel cages with ground corn cobs as bedding. The rabbits

were fed with Laboratory Rabbit Chow (Ralston Purina Co.) and water ad libitum. Rabbits were not maintained under a specific light/dark cycle.

Xenogenous Fertilization Procedure

The procedure for xenogenous fertilization consists of: ovum collection, sperm collection, deposition of gametes in the rabbit oviduct (Rabbit Surgery) and embryo recovery.

Medium: The medium used to supply a safe environment for hamster and squirrel monkey gametes during transfer to and recovery from the rabbit oviduct was a modification of the squirrel monkey in vitro fertilization medium used by Kuehl and Dukelow (1979). The basic contents of the medium used for gamete handling are listed in Table 6. This medium was supplemented with 1 mg/ml hyaluronidase (Sigma Corp., St. Louis, MO) when used for recovery of hamster ova (to remove the follicular cells) and supplemented with 1 unit/ml heparin (The Upjohn Co., Kalamazoo, MI) when obtaining squirrel monkey ova (to prevent clotting). The medium was sterilized by filtering through a 0.45 μm Millipore filter and stored in a sterile Vacutainer at 4°C.

Ovum Collection: Hamster ova were obtained from the oviducts of animals superovulated with an intraperitoneal injection of 30 IU Pregnant Mare Serum Gonadotropin, PMSG (Folligon; Intervet Laboratories; Bar Hill, Cambridge, Great Britain) between 0900 hrs and 1200 hrs followed 56 to 64 hrs later with an intraperitoneal injection of 30 IU Human Chorionic Gonadotropin, HCG (APL; Ayerst Laboratories Inc., New York) (Yanagimachi and Chang, 1964). These hamsters were

TABLE 6

The Basic Contents of the Medium Used in the Manipulation of Squirrel Monkey and Hamster Gametes

Ingredient	Amount	Source
TC 199[a]	80%	GIBCO Laboratories Grand Island, N.Y.
GG Free Fetal Bovine Serum[b]	20%	GIBCO Laboratories Grand Island, N.Y.
Sodium Pyruvate	115.2 μg/ml	Sigma Chemical Co. St. Louis, MO
Gentamicin	0.1 mg/ml	Schering Corp. Kenilworth, N.J.
Penicillin-Streptomycin	100 units and 100 μg/ml	North American Biological Miami, FL

[a]Medium 199 with 25 mM HEPES buffer, Earle's salts and L-Glutamine.

[b]Heated at 56°C for 30 minutes.

sacrificed, by cervical dislocation, 14 to 16 hrs after HCG administration. The oviducts were removed, dissected free of fat and flushed from the fimbrae with 0.3 ml of medium. Mature ova were counted and transferred to the rabbit oviduct.

Squirrel monkey ova were collected by laparoscopic aspiration of follicles (Dukelow and Ariga, 1976) from females after administering a regimen of gonadotropins to induce ovulation (Dukelow, 1970). This ovulatory regime of gonadotropins consisted of injecting intramuscularly for four days 1 mg follicle stimulating hormone, FSH (Burns Biotec, Oakland, CA) with an intramuscular injection of 250 IU HCG on the fourth day (Dukelow, 1979). During the summer months, July to October, five days of i.m. injections of 1 mg FSH were administered due to the seasonality of the squirrel monkey (Harrison and Dukelow, 1973). The squirrel monkeys were then laparoscoped 12 to 18 hrs after the administration of HCG. Follicles were then aspirated with a 25 gauge, 5/8" needle into 0.1 ml medium. The follicular contents were deposited into an 8 chamber tissue culture chamber slide (Lab-Tek, Miles Laboratories Inc., Naperville, IL) and incubated in a 37°C, moist atmosphere with 5% CO_2 in air until transfer to the rabbit oviduct.

Sperm Collection: Hamster sperm was collected from the epididymi of mature males. Hamsters were sacrificed, via cervical dislocation, and their epididymi were dissected. The cauda epididymus was minced in 1 ml of medium. A 0.05 ml aliquot of this solution was then diluted five times and a sample of this solution was evaluated under the light microscope for motility and structural normality.

Semen, obtained by electro-ejaculation of unanesthetized male squirrel monkeys, was collected in 0.5 ml of medium. A sample of this solution was evaluated for motility and structural normality under the light microscope.

Hamster and squirrel monkey sperm suspensions were held in a 37°C water bath until deposited in a rabbit oviduct. Twenty-four hours after collection, the sperm concentration was determined via hemocytometer.

Rabbit Surgery: Adult female rabbits (New Zealand White) were given 100 IU HCG, i.v., to induce pseudopregnancy (Harper, 1963). On the day of surgery, each rabbit was anesthetized with 60 mg/2.25 kg body weight of sodium pentobarbital (Nembutal^{R}, Abbott Laboratories, North Chicago, IL) followed by ether inhalation to maintain a surgical plane of anesthesia. The reproductive tract was then exposed through a 7 cm mid-ventral incision. Ova, in 5 µl aliquots were deposited using a micropipetter^{R} (SMI, Scientific Manufacturing Industries, Emeryville, CA) into the fimbriated end of the ampulla. Depending on the number of ova, 1 to 4 aliquots were deposited.

After deposition of ova into the oviduct, 0.05 ml of conspecific sperm solution was deposited. Sperm were deposited using a 0.25 ml tuberculin syringe, fitted with a 20 gauge, 1.5" needle to which 5 cm polystyrene tubing (0.034 inches id, 0.050 inches od; Clay Adams, Parsippany, NJ) was affixed. After insemination the tubal uterine junction was ligated with 00 gut suture and the incision closed.

Embryo Recovery: Each rabbit was killed by cervical dislocation and the reproductive tract was removed. Using a 5 ml syringe

with blunted 25 gauge, 5/8" needle, medium (2 ml) and air (1.5 ml) was flushed through the oviduct from the uterine end. The oviductal contents were collected in a watch glass and under a dissecting microscope recovered embryos were observed. Fertilization was judged to have occurred if 2 polar bodies and 2 pronuclei were observed or if cleavage had occurred.

Statistical Analysis: All data were analyzed using the Chi Square test for homogeneity. Significance was judged at the alpha = 0.05 or 0.01 level.

RESULTS

A total of 585 hamster ova was deposited into oviducts of pseudopregnant rabbits and 198 (33.8%) were recovered. Of the recovered ova, 119 (60.1%) were fertilized and 38 (31.9%) of the fertilized ova developed to the two cell stage. A total of 79 (38.4%) squirrel monkey ova were recovered from among 206 ova deposited in rabbit oviducts. Of those recovered ova, 25 (31.6%) were fertilized and 3 (12.0%) of the 25 had developed to the two cell stage.

Xenogenous fertilization of mouse ova in oviducts of pseudopregnant rabbits yielded 36 (19.8%) recovered ova from the 182 ova deposited in the rabbit oviduct. Of the 36 recovered ova 4 (11.1%) were fertilized. No cleavage of mouse ova was observed and because of the lack of cleavage, attempts to achieve xenogenous fertilization with mice were terminated.

The effects of the day of pseudopregnancy on the ability of the rabbit oviduct to support fertilization of hamster and squirrel monkey was investigated and the results for hamster and squirrel monkey ova can be found in Table 7.

Day of pseudopregnancy had no significant effect on the xenogenous fertilization rate of hamster or squirrel monkey ova (χ^2 = 6.81 and 0.13). Cleavage of hamster and squirrel monkey ova, also, were not effected by day of pseudopregnancy (χ^2 = 1.84 and 1.33).

TABLE 7

Xenogenous Fertilization of Hamster and Squirrel Monkey Ova Deposited into the Rabbit Oviduct During Different Stages of Pseudopregnancy

Ovum Donor	Day of Pseudopregnancy	No. of Rabbits	No. of Ova Deposited	No. of Ova Recovered (%)	No. of Ova Fertilized (%)	No. of Ova Cleaved (%)
Hamster	1	7	372	108 (29.0)	59 (54.6)	16 (27.1)
Hamster	2	1	75	50 (66.7)	36 (72.0)	13 (36.1)
Hamster	4	1	58	23 (39.7)	12 (52.2)	4 (33.3)
Hamster	7	1	15	10 (66.7)	6 (60.0)	2 (33.3)
Hamster	10	1	65	7 (10.8)	6 (85.7)	3 (50.0)
Squirrel Monkey	1	23	110	52 (47.3)	16 (30.8)	2 (12.5)
Squirrel Monkey	2	9	59	14 (23.7)	5 (35.7)	0 (0)
Squirrel Monkey	3	5	37	13 (35.1)	4 (30.8)	1 (25.0)

The effects of sperm concentration on the xenogenous fertilization of hamster and squirrel monkey ova were examined and can be found in Table 8. Sperm concentration had no significant effect on the xenogenous fertilization of hamster and squirrel monkey ova (χ^2 = 4.86 and 1.66). Cleavage rates of hamster ova also were not affected by sperm concentration (χ^2 = 3.47).

The effects of time of recovery after deposition in the rabbit oviduct of hamster and squirrel monkey ova are shown in Table 9. The time of recovery from the rabbit oviduct had no significant effect of the xenogenous fertilization of hamster and squirrel monkey ova (χ^2 = 7.51 and 2.64). Cleavage of hamster and squirrel monkey, also, was not effected by recovery time (χ^2 = 2.10 and 1.10).

The effects of the number of hamster ova deposited in the rabbit oviduct on the xenogenous fertilization rates can be found in Table 10. The number of ova deposited into the oviducts of pseudopregnant rabbits had no effect on the xenogenous fertilization or cleavage of hamster ova (χ^2 = 7.34 and 1.88).

The effects of day of pseudopregnancy, sperm concentration, time of recovery and number of ova deposited in the oviduct of the rabbit on ovum recovery were not tested for statistical significance. This was largely due to the confounding effects of experimental technique. It was assumed, in the testing of the significance of these factors on fertilization, that there was no differential recovery of fertilized and unfertilized ova.

TABLE 8

The Effect of Sperm Concentration on the Xenogenous Fertilization of Hamster and Squirrel Monkey Ova

Ovum Donor	Sperm Concentration (sperm/ml)	No. of Rabbits	No. of Ova Deposited	No. of Ova Recovered (%)	No. of Ova Fertilized (%)	No. of Ova Cleaved (%)
Hamster	10^6	2	121	59 (48.8)	33 (55.9)	7 (21.2)
Hamster	10^7	2	97	33 (34.0)	18 (54.5)	5 (27.8)
Hamster	10^8	4	240	37 (15.4)	26 (70.3)	11 (42.3)
Hamster	10^9	1	75	50 (66.7)	36 (72.0)	13 (36.1)
Squirrel Monkey	10^6	7	33	11 (33.3)	5 (45.5)	---
Squirrel Monkey	10^7	14	65	21 (32.3)	5 (23.8)	---
Squirrel Monkey	10^8	10	65	28 (43.1)	8 (28.6)	---

TABLE 9

Xenogenous Fertilization and Development of Hamster and Squirrel Monkey Ova at Varying Times of Recovery After Insemination

Ovum Donor	Recovery Time, hrs After Insemination	No. of Rabbits	No. of Ova Deposited	No. of Ova Recovered (%)	No. of Ova Fertilized (%)	No. of Ova Cleaved (%)
Hamster	28	2	132	52 (39.4)	25 (48.1)	5 (20.0)
Hamster	29	2	130	44 (33.8)	31 (70.5)	11 (35.5)
Hamster	30	3	152	67 (44.1)	45 (67.2)	16 (35.6)
Hamster	32	2	119	16 (13.4)	12 (75.0)	4 (33.3)
Squirrel Monkey	12	1	7	6 (85.7)	1 (16.7)	0 (0)
Squirrel Monkey	24-29	6	31	14 (45.2)	4 (28.6)	0 (0)
Squirrel Monkey	30-38	3	18	15 (83.3)	7 (46.7)	1 (6.7)
Squirrel Monkey	48-51	22	130	41 (31.5)	12 (29.2)	2 (16.7)
Squirrel Monkey	>51	4	11	2 (18.2)	1 (50.0)	0 (0)

TABLE 10

The Effects of the Number of Oocytes Deposited in the Pseudopregnant Rabbit Oviduct on the Xenogenous Fertilization of Hamster Ova

Oocytes Deposited per Oviduct	No. of Oviducts	No. of Ova Deposited	No. of Ova Recovered (%)	No. of Ova Fertilized (%)	No. of Ova Cleaved (%)
<20	3	47	16 (34.0)	9 (56.2)	3 (33.3)
20-29	4	93	24 (25.8)	10 (41.7)	3 (30.0)
30-39	8	275	83 (30.2)	59 (71.1)	22 (37.2)
>40	3	133	66 (49.6)	41 (62.1)	10 (24.4)

DISCUSSION

Oviducts of the pseudopregnant rabbit have been shown to support fertilization of porcine and bovine ova (Hirst et al., 1981). In the present study, it was demonstrated that it was also possible with squirrel monkey, hamster and mouse ova. The ability of oviducts of pseudopregnant rabbits to support xenogenous fertilization varies with the species of the ovum donor. Hamster ova were xenogenously fertilized at a rate of 60.1% while porcine ova showed a fertilization rate of only 2.0% (3/148) (Hirst et al., 1981).

Development after fertilization in the rabbit oviduct is also species dependent. Cleavage of hamster and squirrel monkey were observed at rates of 31.9% and 12%, respectively, but no cleavage of mouse ova was observed. The failure of one cell mouse ova to develop in the rabbit oviduct has been reported (Brinster and Ten Broek, 1969). Hamster ova did not develop beyond the two cell stage demonstrating the similarity of the rabbit oviduct system of fertilization to in vitro hamster fertilization in which no development beyond the two cell stage was possible (Yanagimachi and Chang, 1964).

The day of rabbit pseudopregnancy has been shown to have no effect on the xenogenic culture of sheep (Lawson et al., 1972) or mouse (Brinster and Ten Broek, 1969) embryos. Day of pseudopregnancy did effect the development of rabbit morulae transferred to the uteri

of asynchronous recipients. The morulae continued to develop in 3 to 5 day pseudopregnant rabbits but with degeneration of embryos transferred to the uteri of 9 to 11 day pseudopregnant recipients. Development was not retarded, however, when morulae were transferred to the oviducts of day 11 pseudopregnant rabbits (Adams, 1971).

Rabbit oviductal secretions collected from later days of pseudopregnancy and used in in vitro fertilization of rabbit ova showed an increased ability to support fertilization when compared to oviductal secretions from a day 1 pseudopregnant rabbit (Lambert and Hamner, 1975). The day of rabbit pseudopregnancy had no effect on the fertilization of hamster ova from days 1 through 10 nor squirrel monkey ova from days 1 through 3. Thus, the factors that caused a decreased development of rabbit embryos in rabbit uteri from days 9 through 11 or a decreased fertilization in vitro with day 1 oviductal fluid did not affect the xenogenous fertilization of hamster or squirrel monkey ova.

Sperm at a concentration of $2x10^7$ sperm/ml has been shown to be optimal for in vitro fertilization of hamster ova with a decrease in fertilization rate with lesser or greater sperm concentrations (Talbot et al., 1974). Similar results have been reported for mice with optimal sperm concentrations of 10^5 to $6.3x10^5$ sperm/ml (Tsunoda and Chang, 1975). Sperm concentrations has been shown to have no effect on the xenogenous fertilization of squirrel monkey ova for concentrations of 10^6 to 10^8 sperm/ml and no effect on the xenogenous fertilization of hamster ova for the range 10^6 to 10^9 sperm/ml. There appeared to be an increase in the xenogenous fertilization of hamster ova with increasing sperm concentrations but this was not significant.

Kuehl and Dukelow (1979) reported the time of extrusion of the second polar body after *in vitro* fertilization of squirrel monkey ova was 6 to 22 hours after insemination. The first cleavage occurred 20 to 40 hours after insemination. This rate of development is comparable to that of ova fertilized *in vivo* of other non-human primates (*Macaca mulatta* and *Papio cyncephalus*) and man. This indicates normalcy of development of *in vitro* fertilized ova. The xenogenous fertilization of squirrel monkey ova also demonstrates this normalcy of development, i.e., extrusion of the second polar body occurs at 12 to 48 hrs and first cleavage at 31 to 48 hours. The cleavage rate, however, following xenogenous fertilization was low. Because of the recovery from the rabbit oviduct only at discrete times, the precise time of achieving a developmental stage cannot be precisely determined.

The extrusion of the second polar body and the first cleavage of hamster ova occurs at 6 hours and 24 to 36 hours postcoitus, respectively (Beaty, 1956). An attempt to determine the cleavage time of hamster ova during xenogenous fertilization showed no difference in cleavage rates between 28 to 32 hours. Therefore, the time of cleavage of xenogenously fertilized hamster ova is at least 28 hours and this falls within the time of normal development.

Niwa *et al*. (1980) observed increased in the percent of ova fertilized, *in vitro*, when there was a concomitant increase in the number of ova present. This effect was not observed with xenogenous fertilized hamster ova. The ova, however, were stripped of their cellular vestments with hyaluronidase and any follicular components were

diluted within the rabbit oviduct. The effect of ovum number observed by Niwa et al. (1980) was due, most likely, to the follicular constituents and not the ova themselves.

The oviducts of pseudopregnant rabbits can support the fertilization of squirrel monkey and hamster ova. This process may be utilized to generate embryos which can be utilized for the benefit of mankind.

SUMMARY AND CONCLUSIONS

Squirrel monkey and golden hamster ova were fertilized in the oviduct of the pseudopregnant rabbit. Fertilization rates for squirrel monkey and hamster ova of 25/79 (31.6%) and 119/198 (60.1%) and cleavage rates of 3/25 (12.0%) and 38/119 (31.9%) were observed, respectively. There was no significant effect of the day of pseudopregnancy on the xenogenous fertilization of squirrel monkey and hamster ova. Hamster ova deposited in oviducts of rabbits during days 1, 2, 4, 7 and 10 of pseudopregnancy yielded fertilization rates of 59/108 (54.6%); 36/50 (72.0%), 12/23 (52.2%), 6/10 (60.0%) and 6/7 (85.7%), respectively. Squirrel monkey ova deposited in the oviduct of pseudopregnant rabbits during days 1, 2 and 3 yielded fertilization rates of 16/52 (30.8%), 5/14 (35.7%) and 4/13 (30.8%), respectively.

Sperm concentrations of 10^6, 10^7, 10^8 sperm/ml showed no significant difference on fertilization of hamster and squirrel monkey ova in the pseudopregnant rabbit oviduct. Hamster ova were fertilized at rates of 33/59 (55.9%), 18/33 (54.5%), 26/37 (70.3%) and 36/50 (72.0%) for sperm concentrations of 10^6, 10^7, 10^8 and 10^9 sperm/ml, respectively. Squirrel monkey ova were fertilized at rates of 5/11 (45.5%), 5/21 (23.8%) and 8/28 (28.6%) for their respective sperm concentrations.

The time of cleavage of hamster ova after insemination in the rabbit oviduct was at least 28 hours and at least 31 hours for the squirrel monkey. The developmental times for hamster and squirrel monkey embryos followed normal *in vivo* developmental times.

The number of hamster ova deposited into the rabbit oviduct did not affect the xenogenous fertilization rates or cleavage rates.

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APPENDIX A

APPENDIX A

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APPENDIX B

APPENDIX B

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